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Regulation of MST complexes and activity via SARAH domain modifications

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Abstract

Three elements of the Hippo tumor suppressor pathway – MST1/2, SAV1, and RASSF1–6 – share in common a C-terminal interaction motif termed the SARAH domain. Proteins containing this domain are capable of self-association as homodimers and also of trans-association with other SARAH domain containing proteins as well as selected additional proteins that lack this domain. Recently, the association of MST1/2 with itself or with other proteins has been shown to be regulated by phosphorylation at sites near or within the SARAH domain. In this review, we focus recent findings regarding the regulation of such MST1/2 interactions, with an emphasis on effects of these events on Hippo pathway activity.

Introduction

The Hippo pathway was originally discovered in *Drosophila* from genetic screens designed to identify pathways regulate tissue overgrowth (1–3). These screens revealed that inactivation of genes termed Wrts, Hpo, Mats, and Sav all caused a similar organ overgrowth phenotype, consistent with the idea that all these genes are part of a common developmental pathway. These *Drosophila* genes proved to have close orthologues in mammalian cells, implying a central, conserved signaling pathway in eukaryotes. The canonical core Hippo pathway consists of a two sets of protein kinases, two scaffolding proteins, two co-transcritional regulators, and a family of transcription factors. When the Hippo pathway is activated, these signaling elements act in series: MST1 and MST2 (*a.k.a.* STK4 and STK3; Hpo in Drosophila), in complex with the regulatory protein SAV1 (Sav), phosphorylates a second pair of kinases, LATS1 and LATS2 (Wrts); activated LATS1/2, along with regulatory protein MOB1 (Mats), then phosphorylates the transcriptional co-activators YAP1 (Yki) and TAZ, creating a docking site for 14-3-3 proteins; and the 14-3-3/phospho-YAP1 complex is sequestered in the cytoplasm where it is ubiquitinated and degraded. When the Hippo

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Author Contribution

S.K. and D.B. proposed the subject of the review. S.K. and D.B wrote the initial drafts, and J.C edited the manuscript with input from all authors.

Competing Interests

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pathway is inactive, YAP1/TAZ is underphosphorylated and can enter the nucleus, where it interacts with the TEAD (Sd) family of transcription factors, promoting the expression of genes associated with cell survival and proliferation. More recently, the mammalian Hippo pathway has been shown to include several LATS1/2 activating kinases such as MAP4Ks and TAOKs that can act in parallel to MST1/2, implying increased complexity in higher eukaryotes (4, 5). The particular kinase(s) that phosphorylate LATS1/2 depend on the nature of the stimulus and cell type (5, 6). For example, in HEK293 cells, MST1/2 represents the major LATS activating kinase under conditions of osmotic stress and heat shock (6, 7), but MAP4Ks and/or TAOKs appear to play an equal or greater role under conditions of serum starvation and contact inhibition (4, 5).

In addition to these core signaling elements, various members of the Hippo pathway associate with and are regulated by a number of other proteins. Among the best studied of these inputs are Merlin (the product of the NF2 (neurofibromatosis-2) gene), which facilitates the translocation of LATS to the plasma membrane; Angiomotin, which acts as a scaffold to link LATS1/2 to its activator SAV1-MST1 and its target YAP1 (8, 9); the STRIPAK complex, which targets the protein phosphatase PP2A to MST1/2 and MAP4K4, deactivating these kinases (10–12); and certain members of the RASSF family, which interact with MST1/2, linking the Hippo pathway to RAS-mediated apoptotic signaling in response to DNA damage and replication stress (13–17). In the case of RASSF1A, which is encoded by one of the most commonly epigenetically silenced genes in cancer, binding to MST1/2 prevents its dephosphorylation and also recruits Caspase-3 to the complex, resulting in cleavage of the autoregulatory C-terminus from MST1/2. These events lead to MST1/2 activation and translocation to the nucleus (18–20). Active, nuclear MST1/2 is thought to induce apoptosis by phosphorylating Jun N-terminal kinase (JNK) and also Ser14 on Histone 2B, promoting chromosome condensation and DNA fragmentation (21, 22)

Notably, three components of the Hippo pathway – MST1/2, SAV1, and RASSF1–6 – contain a ~50 amino acid protein-protein interaction module termed the SARAH (**Sa**vador/**Ra**ssf/**H**ippo) domain (Fig. 2C). This coiled-coil domain mediates dimerization between SARAH-domain containing proteins. Structurally, the SARAH domain of MST1/2 is comprised of a short h1 helix (433–437 in MST1), a linker (437–440 in MST1), and a longer h2 helix (441 to 480 in MST1) (23). All three SARAH-domain proteins can form homodimers as well as heterodimers with one another, as well as with other proteins that lack this domain (22–26). How this process is regulated, and the consequences of these diverse interactions, are the subjects of this review.

Homotypic and heterotypic SARAH domain complexes

MST1/2, RASSF1–6, and SAV1 contain SARAH domains that usually mediate homodimerization of these proteins with themselves and heterodimezation with one another (Fig. 1A). In most such complexes, the SARAH domain from one partner engages the SARAH domain of the other in an antiparallel fashion, stabilized by a series of hydrophobic interactions between multiple conserved residues in each domain (23, 27). Homodimerization is thought to a key factor in MST1 and MST2 activation, as the geometry of the homodimeric form facilitates transphosphorylation of a key Thr residue in the

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opposing kinase's activation loop (27). Under *in vitro* conditions, when a non-catalytic SARAH protein such as RASSF5 heterodimerizes with MST1, transphosphorylation cannot occur and MST activity remains low. Indeed, a recent study showed that it is not the SARAH domain *per se* that is required for MST activation, but rather the proximity of the MST kinase domains to one another that ensues upon homodimerization. Such proximity and activation can be brought by other means such as attachment of artificial dimerization motifs or by physiologic events such as recruitment to the plasma membrane (28).

Depending on the MST dimerization partner, the Hippo pathway can be activated or inactivated and consequently induce either cellular apoptosis or proliferation. For example, while MST1/MST1 and MST2/MST2 homodimers are active, MST1/MST2 heterodimers are relatively inactive, and, in this latter form, are associated with YAP activation and oncogenic transformation (25, 26, 29, 30). The structural basis for the inactivity of MST1/MST2 heterodimers is not known, but presumably involves a change in the orientation of the two kinase domains such that autophosphorylation *in trans* is ineffective. The interaction of MST proteins with other SARAH-domain containing dimerization partners has variable effects. For example, the interaction of MST1 or MST2 with RASSF proteins has been shown to positively or negatively regulate MST1/MST2 kinase activity, depending on the particular RASSF isoform, the prior state of MST activity, and the cellular context (29, 31). The dimerization of MST1/2 with RASSF1A activates the protein kinase activity of MST and promotes apoptosis (22, 32–34). The mechanism underlying this event is thought to be RASSF1A-mediated inhibition of MST dephosphorylation by PP2A, a protein phosphatase that is a member of the STRIPAK regulatory complex (20, 35, 36). A similar protective mechanism may underlie the activation of MST1 by SAV1 binding (37). In contrast, the activation of MST1/2 by RASSF2 is thought to be related to stabilization of the MST2 protein by protection from degradation. If RASSF2 is absent, proapoptotic stimuli induce the rapid degradation of MST2, nullifying its effects as a proapoptotic protein (38). Finally, as noted above, while RASSF5 can act as a negative regulator of MST2, it only does so if it binds to MST2 before MST activation loop phosphorylation. This finding can be explained by a model proposed by Ni *et al.*, which posits that RASSF5/MST heterodimers prevent autophosphorylation of MST at its activation loop. In this setting, by binding to unautophosphorylated MST2, RASSF5 blocks MST2 activation and inhibits apoptosis (29).

In canonical Hippo signaling, SAV1 homodimerizes, and these homodimers can heterodimerize with MST1 and/or MST2, forming a heterotetrameric complex that is required for activation of the downstream LATS/MOB complex (24, 37). It is interesting that SAV1 self-association is not mediated by its SARAH domain, as might be expected by a casual consideration of its domain architecture, but rather by an unusual extended tandem WW2 domain (termed WW12ex) (24, 39). Mutations in this region are found in various human cancers and these were shown to disrupt SAV1 homodimerization. In addition, substitution of a phosphomimetic Glu residue for Tyr273 within this domain abrogated SAV1 homodimerization and its ability to activate MST1. It is not known whether this site is subject to phosphoryatlion in cells, but, if so, this might provide another means to impede Hippo pathway activation. A nearby residue, Ser269, as well as three additional Ser residues, have been reported to be phosphorylated by MST2, raising the possibility of a feedback inhibition mechanism (40). Substitutions of Ala residues at these sites resulted in loss of

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SAV1 self-association and loss of MST1 activity, consistent with the idea that the formation and/or stability of this signaling module is regulated by phosphorylation, perhaps as part of a feedback loop (40).

Interestingly, the SARAH domain can in some cases mediate interactions with non-SARAH domain proteins (Fig. 1B). For example, the association of RASSF1A with the mitotic kinase AURA is thought to be mediated via a non-canonical interaction of the helical RASSF1A SARAH domain with the globular kinase domain of AURA (41). Additional proteins that lack a SARAH domain, such as RAF1, can also bind to the SARAH domain of MST1/2 and regulate MST kinase activity (42). In this instance, binding of RAF1 is thought to prevent MST activation by two mechanisms: by engaging the SARAH domain, it prevents MST from homodimerizing, and by bringing a RAF1-associated protein phosphatase into close proximity, it augments dephosphorylation of a key Thr residue in the MST activation loop (25, 43). These and other examples confirm the importance of both homotypic and heterotypic SARAH domain interactions in the regulation of these kinases (32).

Regulation of MST dimerization

An important unresolved question in the field is how SARAH domain-containing proteins select their dimerization partners, and whether they can signal as monomers. Regarding the latter question, it is not clear if MST1 or MST2 are stably present in cells as monomers. Structural analyses suggest that a monomeric SARAH domain would comprise 2 or 3 shorter helices, unlike the dimeric conformation in which there is a single, approximately 45° angle, between the short N-terminal h1 helix and the elongated h2 helix (44, 45). Such isolated monomeric SARAH domains have been shown to be unstable (44, 46). When purified from cells by sizing columns, MST1 appears as a dimer, with little to no protein eluting at its monomeric molecular mass (18). On the other hand, it is possible to express non-dimerizing mutants of MST1, such as MST1 L444P (18), indicating that a mutant, monomeric form of the MST protein is not intrinsically unstable

As indicated above, MST1 and MST2 have been shown to bind to themselves, each other, SAV1, and various RASSF proteins. Given that it is unlikely that MST1 or MST2 can bind to more than one partner at a time via the SARAH domain, some mechanism(s) must exist to regulate this process. For mammalian proteins, it is known that the intrinsic affinity of SARAH domains varies, with a range from low nM (RASSF5/MST1 heterodimers) to mid nM (MST1/MST2 heterodimers) to low μM (MST1/MST1 and MST2/MST2 homodimers). For the Drosophila versions of these proteins, the Sav/Hpo SARAH domain heterodimerization complex was reported as the most stable, whereas the Sav/Sav SARAH domain homodimer was the least stable, and other combinations (Hpo/Hpo, and dRassF/dRassF homodimers, and Hpo/dRassF SARAH domain heterodimers) had similar, intermediate stabilities (47). It is interesting to note that, in two cases that have been studied in detail, SARAH domain heterodimers appear to be favored over homodimers, sometimes by orders of magnitude (23, 30, 47). Despite these kinetic properties, under many conditions high levels of homodimers can be detected. These finding suggest that intrinsic biochemical affinity is only one factor that determines the choice of dimerization partners for SARAH domain-containing proteins. As the expression of some Hippo pathway elements, in

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particular RASSF1A, is frequently silenced in cancers, the choice of dimerization partner is likely to be regulated at least in part by the relative expression levels of the relevant proteins. In addition, interactions of MST, SAV1, and RASSF proteins are known to be regulated by posttranslational modifications within their SARAH domains (see below). Interactions with other elements of the Hippo pathway are also regulated by phosphorylation. For example, Ni *et al.* showed that the affinity of MOB1 for MST1 or MST2 is diminished, and its affinity for LATS2 is increased, when MOB1 is phosphorylated (48). Finally, interactions with various scaffolding proteins may influence Hippo pathway dimerization partners. For example, a recent report suggests that IQGAP1 is a dynamic interactor of MST2, LATS1 and YAP1 (49), and Angiomotin may serve a similar function.

The role of SARAH domain phosphorylation in the regulation of the Hippo pathway

Several groups have shown that protein kinases affect the activity and dimerization properties of MST1 and/or MST2. In a previous study, we showed that H-RAS induced MST1/MST2 heterodimerization, and that this process required MEK (30). Whether MEK directly or indirectly phosphorylates MST1 and/or MST2 is, however, not known. Liu *et al.* demonstrated that by c-Abl induced disruption of c-RAF/MST2 heterodimers and increasing MST2 homodimerization and activity (50). In this case, the c-Abl phosphorylation site was mapped to Y81 of MST2. Protein kinases that are known to directly phosphorylate residues within the SARAH domain include c-ABL and FGFR4, which phosphorylate Tyr433 in MST1 (51, 52) (MST2 lacks an equivalent Tyr residue at this position); mTORC2, which phosphorylates Ser438 in MST1 (53); and PAR-1b (*a.k.a.* MARK2) which phosphorylates Thr440 in MST1 and the equivalent Ser444 in MST2 (Fig. 2) (54). MST1 Y433 is located within the h1 helix (433–437) of the SARAH domain, whereas S438 and T440 are located in the linker between h1 and h2 (22). Interestingly, the h1 helix is unfolded and the h2 helix adopts an extended conformation when MST1 heterodimerizes with RASSF5, which in turn permis the MST1 catalytic domain to reorient is a more favorable conformation to interact with its substrates (23).

Based on the sequence context around the Ser438 site in MST1, it has been suggested that NEK2, in addition to mTORC2, might catalyze phosphorylation at this site (55). Interestingly, MST2 phosphorylates NEK2 at four sites (S356, S365, T406 and S438) (56, 57), but it is not known if NEK2 can act reciprocally on MST2.

Unbiased large scale affinity purification mass spectroscopy (AP-MS) studies have also identified a number of additional protein kinases that associate with MST1/2 (https://www.ncbi.nlm.nih.gov/gene/6788). In addition to previously identified kinases such as AURB, AKT1, LATS1/2, RAF1, and MARK2, these interactors also include the kinases CDK1, PLK, and RIPK4. While there is no additional experimental evidence for the nature or consequences of these latter interactions, it is interesting to note that MST1/2 contain a number of potential phosphorylation sites compatible with CDK1, PLK, and RIPK4. Directed experiments using non-phosphorylatable residues and/or phosphosite mimics,

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combining with loss of function approaches, may provide further clarification of the potential role of these kinases in Hippo pathway signaling.

Regarding the effects of phosphorylation within the SARAH domain, the literature is sometimes conflicting. For example, the phosphorylation of Y433 by c-ABL has been reported to activate MST1, whereas the phosphorylation of this same site by FGFR4 has been reported to inactivate MST1 (Table 1) (51, 52, 58). These opposite conclusions may be related to additional differentially phosphorylated sites in MST1 and/or different cellular contexts (*i.e*., stress signaling in the case of c-ABL versus mitogenic signaling in the case of FGFR4). Phosphorylation of Ser438 by mTORC2 reportedly inhibits MST1 activity, consistent with the role of mTORC2 in cell proliferation, whereas phosphorylation of MST1 at Thr440 or MST2 at the equivalent site (Ser444) reportedly activates MST. Thus, the effects of phosphorylation near or within the SARAH domain are complex and not well understood at a molecular or structural level. While no crystal structures exist for the MST1 SARAH domain bearing phosphomimics at Y433, S438, or T440, the location of these sites, in particular Y433, suggests that their phosphorylation might perturb interactions of the h1 helix with the h2 helix (Fig. 1A). Alternatively, yet these sites might induce the binding of phospho-readers such as SH2 or PTB-domain containing proteins for pY433, 14-3-3 proteins for pS438, and FHA or WD40-domain containing proteins for T440. In this scenario, binding at these sites might provide steric interference ot diomer formation. Whatever the structural basis may be, it is clear from the published studies that the MST kinases can be regulated via modifications near or within the SARAH domain and that these changes in activity might be related to altered dimerization partners. In the near future, identification of additional kinases that phosphorylate MST1 and MST2, as well as structural models of phosphorylated SARAH domains, will give a more complete picture of how Hippo signaling is regulated by this mechanism.

Can controlling dimerization be exploited therapeutically?

The Hippo pathway presents challenges to therapeutic intervention, as its most readily druggable targets (*i.e*., its kinases including MST1/2, MEKKs, TAOKs, and LATS1/2) are tumor suppressive, whereas the oncogenic elements such as YAP1, TAZ, and the TEAD proteins, are more difficult to target. Currently, TEAD represents the most promising of these targets, as it requires lipid binding for full activity and its lipid binding pocket is accessible to small molecule inhibitors (59–61). However, it is also possible that altering the dimerization of Hippo pathway elements could prove a viable strategy. For example, as noted above, we have shown that active H-RAS induces MST1 to heterodimerize with MST2, and that this complex is relatively inactive. As MEK inhibitors blocked this event, it is conceivable that ERK pathjway blockade could be used to promote the formation or stabilization of highly active, tumor suppressive MST homodimers. Similarly, kinases such as mTORC2 have been shown to reduce homodimerization of MST1, and, by a similar logic, mTORC2 inhibitors might be used to promote the formation of active MST homodimers.

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Abbreviations

AP-MS	Affinity purification mass spectroscopy
AurA	Aurora A
CDK	Cyclin dependent kinase
FGFR	Fibroblast growth factor receptor
LATS	Large tumor suppressor kinase
MAP4K4	Mitogen activated protein kinase kinase kinase kinase
MARK	MAP/microtubule affinity-regulating kinase
MOB	Mps one binder
MST	Mammalian Ste20-like
mTORC	mechanistic target of rapamycin
NEK	NIMA related protein kinase
NF2	Neurofibromatosis-2
PAR	Polarity kinase
PLK	Polo kinase
PP2	Protein phosphatase 2
RASSF	Ras association domain family
RIPK	Receptor interacting protein kinase
SARAH	Salvador/Rassf/Hippo
SAV	Salvador
Sd	Scalloped
STK	Serine/threonine kinase
TEAD	TEA domain family member
YAP	Yes associated protein

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Perspective

- The Hippo tumor suppressor is largely regulated by protein protein interactions, including those between proteins containing SARAH domains. A better understanding of how these interactions are regulated might reveal new therapeutic opportunities.
- The SARAH domain, present in three constituents of the Hippo pathway, is a key element in regulating pathway activity. SARAH-domain interactions are governed not only by intrinsic affinities that are dictated by the specific amino acid sequence of the domain, but also by posttranslational modifications including phosphorylation.
- As new data on posttranslational modifications come to light, we believe that phosphorylation within the SARAH domain will prove to be an important factor in dictating the specificity of its interactions. Identifying the kinases and phosphatases that regulate this process will yield a more dynamic and useful model of Hippo pathway regulation.

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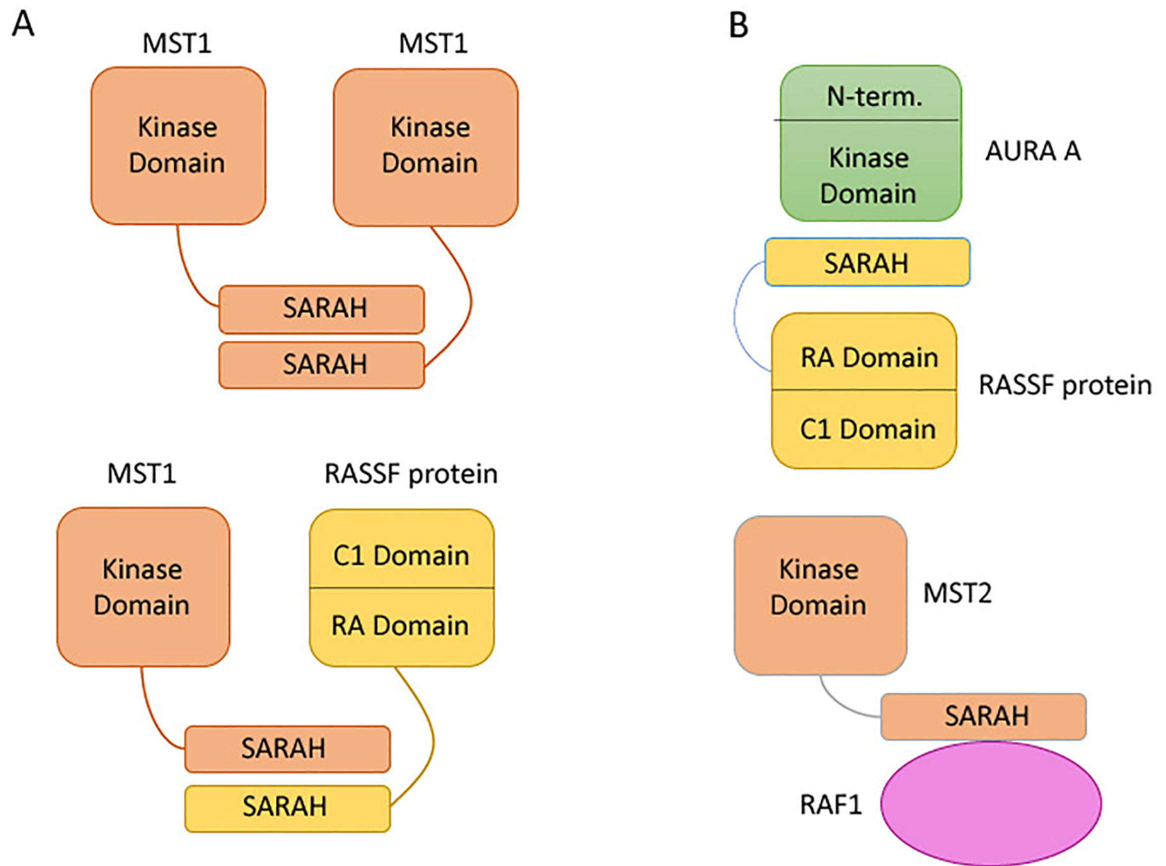

Fig. 1. Complexes between SARAH-domain containing proteins.

(A) Examples of homotypic protein complexes involving MST1 and RASSF1, respectively. **(B)** Examples of heterodimeric interactions involving the SARAH domain of RASSF1A and MST2, respectively, with the kinase domains of Aurora A and RAF1, respectively.

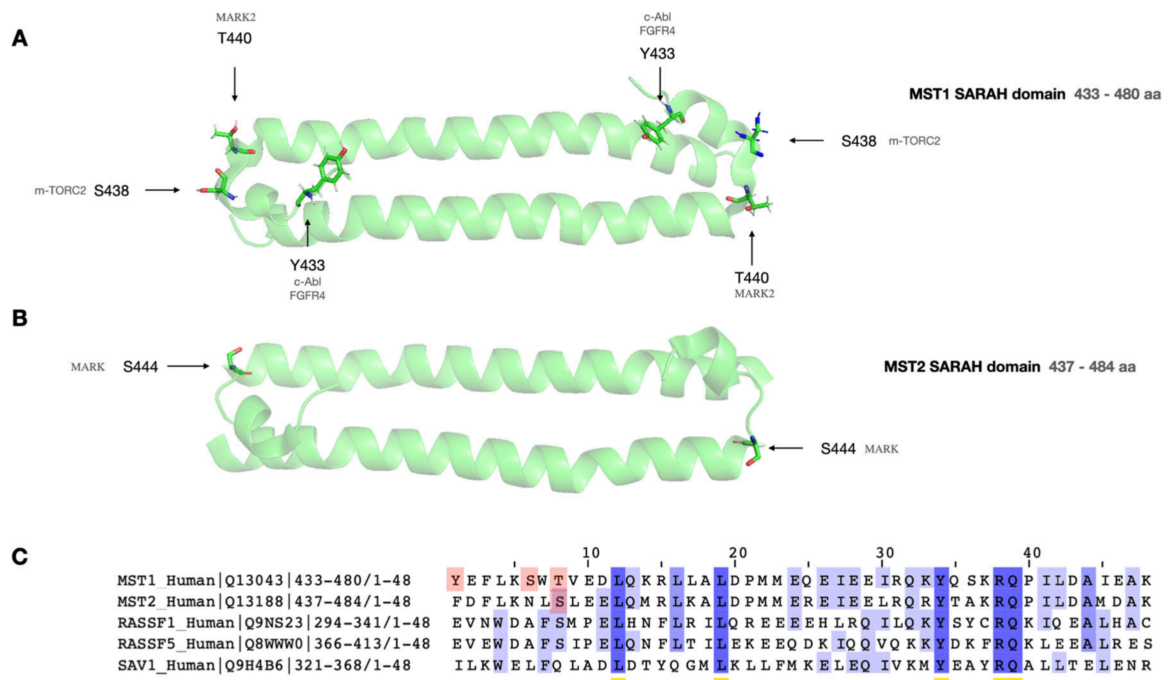

Fig. 2. Cartoon and stick representation of the crystal structure of Human SARAH domain.
(A) Ribbons diagram of the MST1 SARAH domain (green) (PDB ID 2JO8), showing the amino acids Y433, S438 and T440 residues (colored). **(B)** Ribbons representation of the MST2 SARAH domain (green) (PDB ID 4OH9), showing the amino acid S444 residue (colored). The super-secondary structures were prepared using the PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. Note that the SARAH domain is showed as a monomer for clarity, but is derived from the dimeric form. **(C)** Multiple sequence alignment of SARAH domain of MST1, MST2, RASSF1, RSAAF5 and SAV1 proteins. The UniProt (62) accession numbers are shown after the names of the sequences. The conserved residues are indicated by the default blue colouring scheme of the Jalview program. The residues shown in A and B are highlighted with red background. The structural alignment was created in T-Coffee (63) and the picture using Jalview 2 (64).

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Table 1.

MST1 and MST2 C-terminal sites of phosphorylation

Site	Kinase	Target	Effect	Reference
Y433	c-ABL FGFR4	MST1	Activation Inactivation	50,51 52
S438	mTORC2	MST1	Inhibition	53
Thr440/Ser444	PAR-1b (MARK2)	MST1/MST2	Activation	54